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A Search for EGF-Elicited Degradation Products of the EGF Receptor

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Epidermal growth factor (EGF) induces the degradation of EGF receptors in both human foreskin fibroblasts and A-431 cells. Similar degradation products of ^{125}I-EGF covalently linked to its receptor appeared at the same times in both A-431 cells and fibroblasts when the cells were exposed to a concentration of 10 ng/ml EGF. Although the products between the two cell types differed in molecular weight, this was at least partly caused by an actual difference in the receptor proteins from the two cell types (as shown by partial proteolysis) rather than from different pathways of receptor degradation. However, when EGF receptors were biosynthetically labeled, no receptor degradation products could be observed, even when the receptor was labeled with radioactive mannose or phosphate, molecules which would predominantly label the outside or inside face of the receptor, respectively. At 20°C, degradation of the receptor slowed and a 150,000-dalton degradation product was observed. This degradation product has previously been observed in cell homogenates produced in the presence of calcium, mediated by calpain. Thus, calpain may play a role in the intracellular degradation of the EGF receptor.

Key words: epidermal growth factor, protein degradation, membrane protein, tyrosyl kinase, calpain

Epidermal growth factor (EGF) has a number of mitogenic and nonmitogenic effects on several cell types. [1–3]. These effects of EGF presumptively are mediated by the number and/or activity of specific cell membrane receptors that contain an intrinsic tyrosine kinase activity. How the EGF receptor mediates both mitogenic and nonmitogenic stimuli is unclear although much attention has been focused on the role of EGF-stimulated tyrosine phosphorylation of cellular protein [4]. It is also possible that the degradation of the EGF receptor produces one or more fragments that have regulatory significance [5].

Abbreviations used: BSA, bovine serum albumin; CS, bovine calf serum; DM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; Hepes, 4-(2-hydroxyethyl)-1-piperazinethane sulfonic acid; RIPA, 10 mM Tris pH 8.5, 0.15 M NaCl, 3mM, EDTA, 1% SDS, 0.1% sodium azide, and 3 mM iodoacetate; SDS, sodium dodecyl sulfate.

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The possibility that the EGF receptor may be degraded in response to the binding of its ligand was first indicated in a study by Carpenter and Cohen [6] in which the capacity of fibroblasts to bind EGF was decreased if they were previously exposed to EGF. Subsequently, morphological studies showed that both EGF and its receptor may be degraded together since they were both incorporated into lysosomal multivesicular bodies [7–11]. Direct measurements of EGF receptor levels by using antibodies showed that adding EGF to cells induced degradation of its receptor [11–13]. From these studies it was hypothesized that a degradation product of the EGF receptor could be a signal to initiate cellular proliferation. Paradoxically, EGF stimulated normal fibroblasts to divide and grow but inhibited the growth of A-431 cells even though it stimulated the degradation of its receptor in both cell types [12,13]. Therefore, it was of interest to determine if the degradation products of the EGF receptor were different between these two cell types and if these differences could explain the opposite effects of EGF on their cell growth. In this study the production of receptor degradation products was followed after labeling the EGF receptor either by covalently coupling ^{125}I-EGF to its receptor or by biosynthetic incorporation of radioactive mannose, phosphate, or methionine. After EGF is exposed to harsh oxidative conditions, it acquires the capacity to covalently bind to its receptor. In living cells, complexes of EGF covalently bound to its receptor are slowly degraded and have stable polypeptide degradation products [14,15]. Monitoring EGF receptor degradation by using covalent coupling of EGF to its receptor has certain inherent limitations. Modified proteins may be degraded by different degradative pathways than the native protein. Similarly, the covalent addition of EGF to its receptor may cause the receptor to be degraded by the pathways involved in EGF metabolism rather than those for the receptor. Therefore, we also used biosynthetically incorporated radioactive precursors of the EGF receptor followed by immunoprecipitation to follow EGF-induced EGF receptor degradation. The present study showed that (1) cellularly processed degradation products of ^{125}I-EGF receptor complexes differed in fibroblasts and A-431 cells; (2) at least part of this difference can be explained by actual differences in the structure of the EGF receptor between these two cell types; (3) these products were not present in biosynthetically labeled cells under similar conditions, indicating that covalent linkage of EGF to its receptor may modify the degradation pathway of the receptor; and (4) at 20°C a 150k degradation product of the EGF receptor could be observed in response to EGF binding.

METHODS

Cell Culture

Confluent human foreskin fibroblasts (approximately 5×10^5 cells/dish in 60-mm tissue culture dishes (Corning, NY) were prepared essentially as previously described [16]. The growth medium consisted of Dulbecco's modified Eagle's medium (DM) (Gibco Laboratories, Grand Island, NY), pH 7.5, supplemented with 10% calf serum (CS). (Microbiological Associates, Walkersville, MD), 50 mg/liter gentamycin (Gibco), 20 mM 4-(2-hydroxyethyl)-1-piperazinethane sulfonic acid (Hepes), and 10 mg/liter ascorbic acid (Calbiochem-Behring Corp., San Diego, CA). Confluent A-431 cells (approximately 1×10^5 cells/well) in 48-well plates (Costar, Cambridge, MA) were basically prepared as described previously [17]. Their growth medium was the same as that for fibroblasts except that ascorbic acid was omitted and only 5% CS was used.

Before adding radioactive labeling medium, the cells were washed 3 times with Dulbecco's phosphate-buffered saline.

Immunoprecipitations

This procedure was performed essentially as previously described [12,13]. Briefly, the cells were washed 3 times with phosphate-buffered saline, pH 7.3, before solubilization in 1 ml ice-cold RIPA (10 mM Tris, pH 8.5, 0.15 M NaCl, 3 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 0.1% sodium azide, and 3 mM iodoacetate) for 30 min at 4°C. The extracts were centrifuged in a Beckman microfuge B to remove insoluble material. To initiate the immunoprecipitation, 1 μl of anti-EGF receptor serum [18] or 1 μl of normal serum was added to 0.95 ml of the fibroblast extract or to 0.4 ml of the A-431 extract. After incubation at room temperature for 1 hr, 20 μl of a 10% suspension of fixed *Staphylococcus aureus* cells (Zymed Laboratories, Burlingame, CA) was added and the mixture was incubated for an additional 15 min. The immune complexes were precipitated by centrifugation for 1 min in the microfuge and the pellets were washed 3 times with 1 ml RIPA at 37°C and once with 1 ml water. Before use of the fixed *Staphylococcus* cells, they were heated at 95°C for 30 min in an equal volume of 10% β-mercaptoethanol and 3% SDS. This procedure reduced precipitation of cellular proteins in the absence of antiserum, presumable by denaturing *Staphylococcus* proteins (other than protein A) that have specific interactions with mammalian cell proteins.

Electrophoresis and Fluorography

The immunoprecipitated samples and molecular weight markers (either ^{14}C from Bethesda Research Laboratories, Bethesda, MD, or unlabeled from Bio-Rad, Richmond, CA) were prepared for electrophoresis by heating to 95°C for 5 min in Laemmli buffer [12,13]. The samples were electrophoresed on sodium dodecyl sulfate 5–15% acrylamide linear gradient gels unless otherwise specified. The gels were processed for fluorography [19] by using sodium salicylate (Aldrich, Milwaukee, WI) if they contained tritium or ^{35}S. Gels containing ^{32}P were stained with Coomassie Blue and dried prior to autoradiography. Kodak X-Omat AR film (Rochester, NY) was exposed for an appropriate period of time to the dried gels at −70°C by using a Dupont Cronex Lightning-Plus screen (Picker, Nashville, TN). The film was developed in a Kodak RLP X-Omat Processor.

Materials

EGF was isolated from mouse submaxillary glands as described by Savage and Cohen [20]. EGF for covalent coupling was iodinated by the procedure of Comens et al. [21]. Rabbit antiserum 451 to the EGF receptor was previously prepared and characterized [18]. L-[^{35}S]methionine (600–800 Ci/mmol), D-[2−^{3}H (n)]mannose (27.2 Ci/mmol) and [^{32}P]phosphate (1000 Ci/mmol) were purchased from Amersham Corp. (Arlington Heights, IL), New England Nuclear (Boston, MA), and ICN Radiochemicals (Irvine, CA), respectively. All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise specified.

RESULTS

The production of endogenous degradation products of the EGF receptor by normal fibroblasts and A-431 carcinoma cells was investigated. Several degradation products

were identified after covalently coupling ^{125}I-EGF to either fibroblasts or A-431 cells and incubating the live cells at 37°C. The molecular masses of the fragments slightly differed in the two cell types as determined by SDS-PAGE (Fig. 1). In fibroblasts, EGF receptor degradation products had apparent molecular masses of 151k, 135k, 130k, 66k, 54k, 48k, 37k, and 35k. In A-431 cells 151k, 135k, 126k, 79k, 72k, 55k, 42k, 38k, and 35k fragments were identified. These fragments are similar to those previously identified in fibroblasts [14,15,22]. Degradation products progressively became smaller over a period of hours, indicating a slow rate of degradation. Low molecular mass fragments appeared at approximately the same time in the two cells types. Although the receptor fragments differed in electrophoretic mobility in the two cell types, there were a similar number of fragments which did not differ greatly in molecular weight. To determine whether intrinsic differences in the structure of the EGF receptor between the two cell types might explain this phenomenon, [^{35}S]methionine-labeled cellular membranes were subjected to proteolysis in vitro. Similar to the intracellularly processed EGF receptor, the electrophoretic migration of the artificially produced (protease treated) A-431 receptor fragments was slower than that of those from fibroblasts (Fig. 2). In the control lanes the intact fibroblast and A-431 EGF receptors are shown. The immunoprecipitated receptors treated with chymotrypsin (chy) or trypsin (trp) reveal differences in the electrophoretic migration of similar fragments of the two cell types. Several reports have indicated that glycosylation of the A-431 cell EGF receptor is abnormal [23,24]. Thus it is likely that this abnormal glycosylation of the A-431 EGF receptor causes retarded electorphoretic migration of its fragments. Although there are also quantitative differences in the relative amounts of receptor degradation products between the two cell types (Fig. 1), it is not clear whether this observation is important (discussed below).

When the EGF receptor was biosynthetically labeled with [^{35}S]methionine, no fragments were specifically induced by incubation of the cells with EGF at 37°C in either A-431 cells or fibroblasts, even when the autoradiograph was heavily overexposed (Fig. 3, lanes C and O). It should be noted that degradation products of the receptor are visible in this autoadiograph (i.e., 150k, 130k, and 110k); since they are present in both EGF-treated and control cells, they must have been produced either by constitutive turnover of the receptor or during lysis of the cell. It should also be noted that the reduction in number of EGF receptors induced by EGF in A-431 cells (lane O) is masked by the heavy overexposure of the film. Shorter exposure of the film reveals a reduction in number of EGF receptors by EGF (figure inset). Film overexposure also reveals prominent bands which are also precipitated in the absence of specific antibodies to the EGF receptor (lanes A, L, and M). These represent nonspecifically precipitated proteins.

Because no degradation products were observed at 37°C, the cells were treated with inhibitors and at a low temperature in order to slow the rate of degradation. The lysosomal inhibitors, methylamine and chloroquine, did not induce the production of stable degradation products (Fig. 3, lanes G and I). Nor did monensin, an inhibitor of intravesicular traffic (lane K). A 150k fragment was stable when the cell was incubated at 20°C (lane E). Thus processing of the EGF receptor is highly temperature sensitive. Low temperature is thought to inhibit fusion of endosomes with lysosomes [9,10].

Much of the radioactive label may be lost upon proteolysis even if equimolar levels of fragments of the receptor are present because methionine residues are present throughout the entire EGF receptor molecule [25]. To circumvent this problem, [2-^{3}H]mannose was used to biosynthetically label the EGF receptor (Fig. 4). This sugar is incorporated into the carbohydrates located on the extracellular face of the receptor [4]. Thus, this

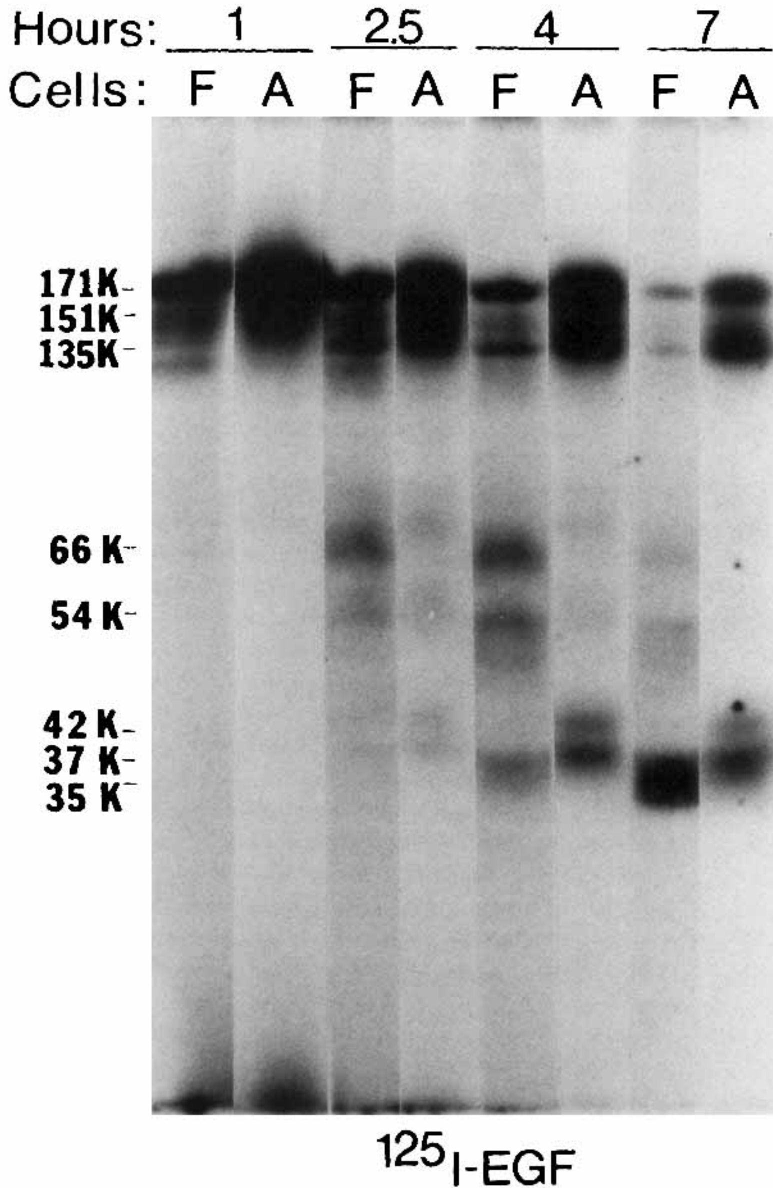

Fig. 1. Comparison of cellularly metabolized degradation products of covalently linked ^{125}I-EGF: EGF receptor complexes in fibroblasts and A-431 cells. Fibroblasts and A-431 cells were incubated with 10 ng/ml ^{125}I-EGF in binding buffer (0.1% BSA in DM) for 1 hr at 37°C. The labeling solution was removed and replaced with buffer without EGF and the cells were incubated at 37°C until the times indicated in the figure. After incubation, the washed cells were scraped into 0.15 ml Laemmli buffer containing 1 mM EDTA and the extract was heated at 95°C for 15 min. The extracts were placed on 5–15% gradient SDS gels and the gels were processed as described in Methods. The film was exposed 2 days for A-431 cells and 2 wk for fibroblasts. The lanes are labeled **F** for fibroblast extracts or **A** for A-431 extracts.

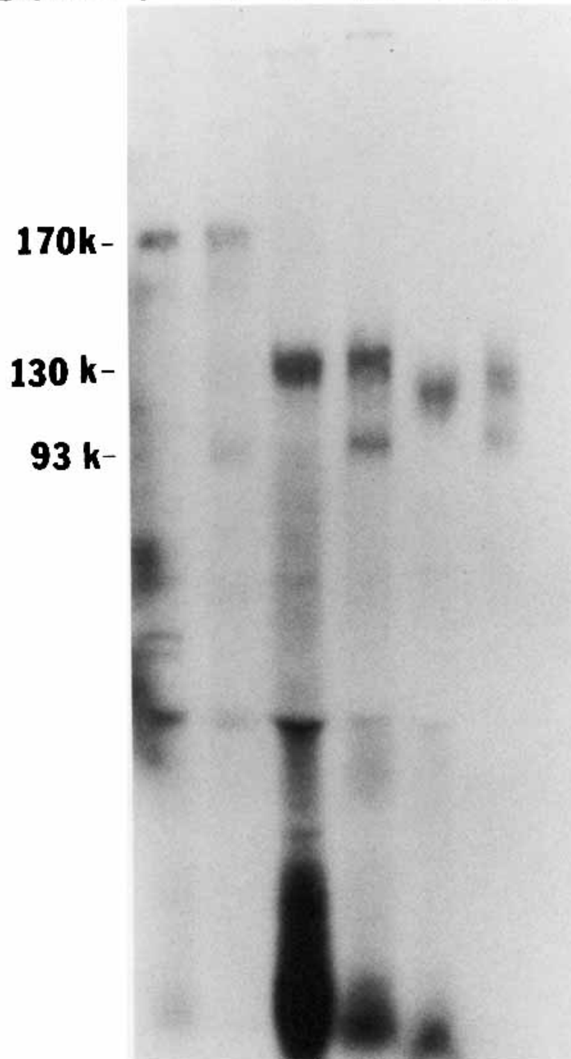

Fig. 2. Comparison of partial proteolytic fragments of the EGF receptor. Fibroblasts (ten 60-mm dishes) were biosynthetically labeled overnight with 25 μCi/ml [^{35}S]methionine in 10% CS in methionine-free minimum essential medium (Flow, McLean, VA). A-431 cells (one 2-cm^2 well) were labeled 1 hr with 50 μCi/ml [^{35}S]methionine and incubated for 4 hr without the label before harvesting the cells. The EGF receptor was immunoprecipitated from the pooled cellular extracts with 10 μl of antiserum as described in Methods and the immunoprecipitate was washed twice with 20 mM Hepes buffer, pH 7.4, before suspending them in 0.1 ml 20 mM ammonium bicarbonate buffer, pH 7.8. The immunoprecipitates were incubated with 0.5 mg/ml chymotrypsin (**chy**) or trypsin (**trp**) for 1 hr at 24°C after which Laemmli buffer was added and the fibroblast (**lanes F**) and A-431 (**lanes A**) extracts were electrophoresed on a 5–15% gradient gel. Four times as much cell extract was used in the protease-treated lanes in order to compensate for the loss of receptor protein due to proteolysis. The gel was processed as described in Methods. The film was exposed for 2 wk at −70°C.

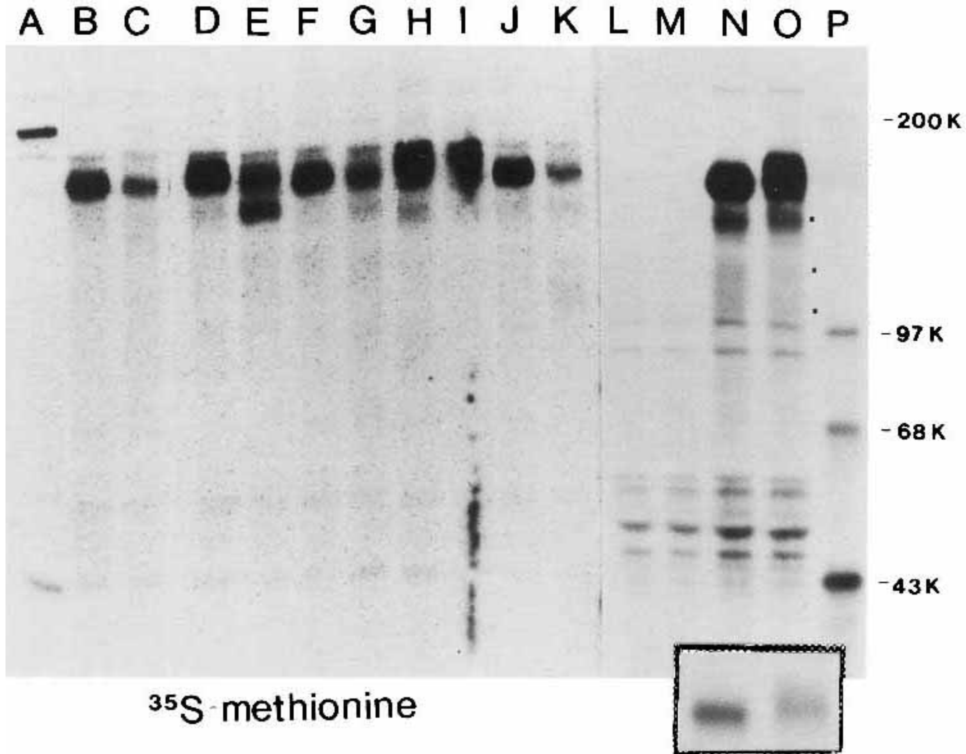

Fig. 3. Immunoprecipitations from [^{35}S]methionine-labeled cells. Fibroblasts were incubated 18 hr with 20 μCi L-[^{35}S]methionine in 1.5 ml methionine-free minimum essential medium containing 10% CS. After removing the labeling medium, the cells were incubated for 1 hr at 37°C in the presence of 10mM methylamine (F and G), 0.2 mM chloroquine (H and I) or 0.02 mM monensin (J and K) in 0.1% BSA in DM. The cells were incubated for 4 more hr in the absence (**A**,**B**,**D**,**F**,**H**, and **J**) or presence (**C**,**E**,**G**,**I**, and **K**) of 10 ng/ml EGF. The immunoprecipitates shown in lanes D and E were from cells incubated at 20°C. A-431 cells (**L–O**) were incubated 2 hr with 20 μCi L-[^{35}S]methionine in 0.2 ml 10% CS in methionine-free MEM. After removing the labeling medium, the cells were incubated for 2 hr in 10% CS in DM, washed, and then incubated in the absence (L and N) or presence (M and O) of 200 ng/ml EGF. Fibroblasts and A-431 cells were prepared for immunoprecipitation and electrophoresis of the immunoprecipitates produced as described in Methods. All immunoprecipitations were performed with antiserum to the EGF receptor except for those shown in A, L, and M, where the antiserum was replaced with normal serum. The film was exposed to the dried gel for 1 wk in the case of the A-431 cells and 4 wk for the fibroblasts. The **inset** below lanes N and O shows a room temperature, 3-day-exposed film of the same gel as used to obtain the fluorograph shown above. Only the area corresponding to the undegraded EGF receptor from lanes N and O is shown. Dots on the right-hand side of lane O show the location of the 150, 130, and 110k degradation products of the EGF receptor in A-431 cells.

label should monitor the fate of the same domain to which ^{125}I-EGF becomes linked. Proteolytic fragments were not detectable by using this label (lanes D, H, and J). The whole A-431 cell extract was placed in lanes I (control) and J (EGF exposure) as a control to determine whether nonimmunoprecipitable fragments might be observed. The EGF receptor is the major labeled protein and composes 5% of the mannose-labeled macromolecules in A-431 cells [13]. No bands unique to the EGF-treated cells were

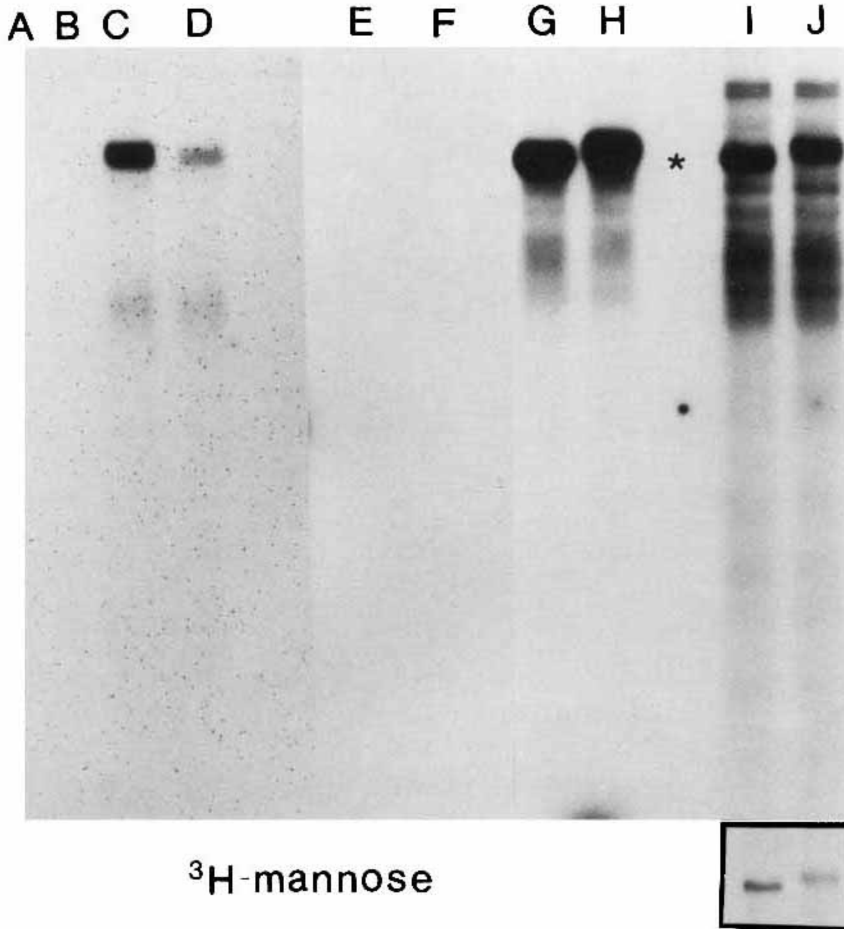

Fig.4. Immunoprecipitation from [^{3}H]mannose-labeled cells. Fibroblasts (**lanes A–D**) were incubated 18 hr with 60 μCi [2-^{3}H]mannose in 1.5 ml glucose-free DM containing 10% CS. After removing the labeling medium, the cells were incubated for 4 hr at 37°C in 1% BSA in DM plus (B and D) or minus (A and C) 10 ng/ml EGF. A-431 cells (**lanes E–J**) were incubated 18 hr with 20 μCi [2-^{3}H]mannose in 0.2 ml glucose-free DM containing 10% CS. After removing the labeling medium, the cells were incubated for 3 hr at 37°C in 1% BSA in DM plus (F, H, and I) or minus (E, G, and J) 200 ng/ml EGF. Fibroblasts and A-431 cells were prepared for immunoprecipitation and the immunoprecipitates were electrophoresed as described in Methods. After processing and drying the gel, it was placed on film for 8 days (A-431 cells) or 4 wk (fibroblasts). Lanes A, B, E, and F were from immunoprecipitations containing antiserum to the EGF receptor. Lanes I and J were whole cell extracts. An asterisk indicates the location of the EGF receptor on the gel. The **inset** below lanes I and J shows a 1-day-exposed film of the same gel as used to obtain the fluorograph shown above. Only the native EGF receptor region from lanes G and H is shown.

observed, confirming the contention that no stable mannose-labeled degradation products are produced in response to EGF binding. It should be emphasized here that some of the bands shown in lanes I and J are unrelated to the EGF receptor because the total cellular protein was applied to these lanes.

One possible reason that fragments of the EGF receptor cannot be detected in biosynthetically labeled cells is that the antiserum does not recognize low molecular weight fragments. Figure 5 shows that the antiserum has the capacity to precipitate ^{125}I-EGF/EGF receptor covalent complexes of the type shown in Figure 1. In this figure, several fragments including the smallest 37k fragment were immunoprecipitated (lane B).

To further assess the capacity of the antiserum to precipitate EGF receptor fragments, the EGF receptor was labeled by using a variety of radioisotopes and subjected to proteolytic digestion. This is an important experiment because it eliminates the possibility that the antiserum in the experiment shown in Figure 5 only recognized the EGF

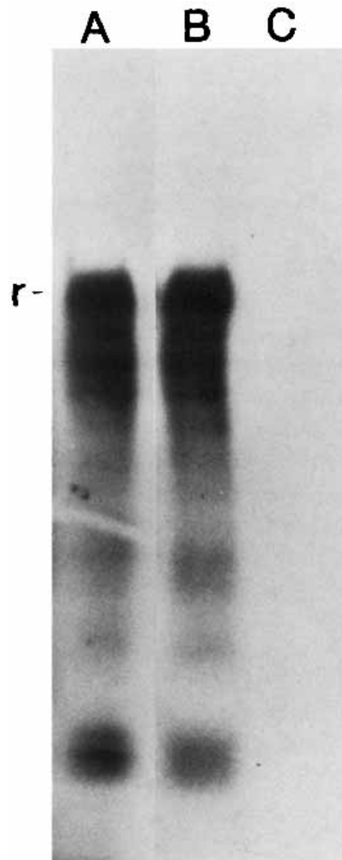

Fig. 5. Immunoprecipitation of EGF covalent complexes. A-431 cells were incubated with ^{125}I-EGF for 4 hr as described in Figure 1. **Lane A** exhibits the radioactive profile of proteins solubilized in 220 μl Laemmli buffer and **lanes B** and **C** are immunoprecipitations of RIPA solubilized cells utilizing 5 μl antiserum (lane B) or 5 μl normal serum (lane C). The samples were electrophoresed and the film was exposed to the dried gel for 5 (lane A) or 10 days (lanes B and C) at −70°C by using a Lightning-Plus screen. **r** indicates the position of the native EGF receptor.

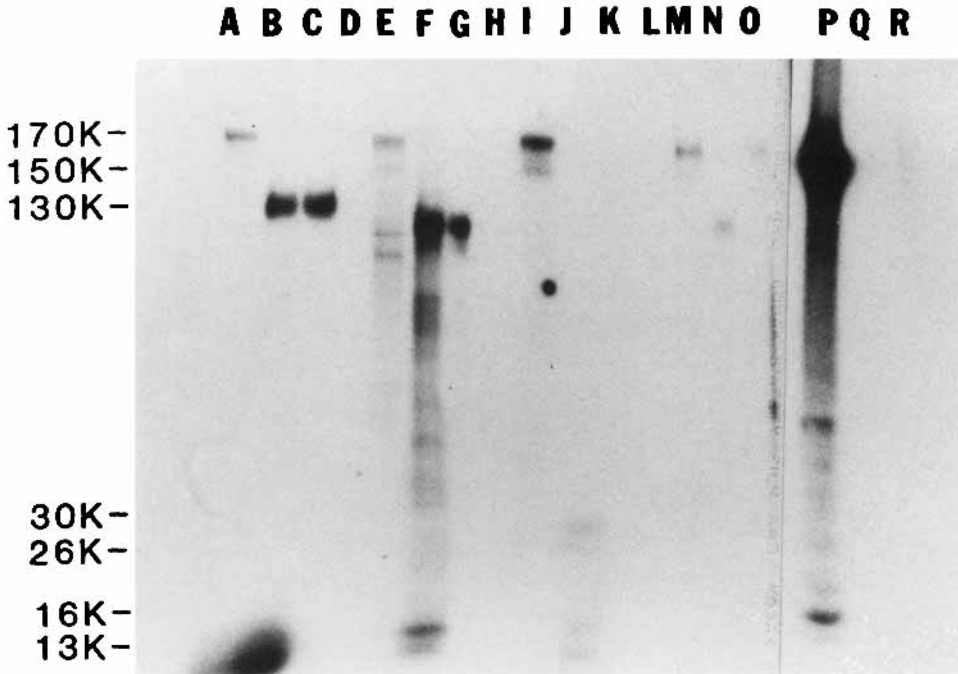

Fig. 6. Recognition of EGF receptor fragments by antisera. To label EGF receptors with ^{125}I-EGF (**lanes A–D**) or [^{32}P]ATP (**lanes I–L**), washed, confluent A-431 cells in 24-well cluster plates were scraped into 1 ml of ice-cold phosphate-buffered saline containing 3 mM EDTA (one well each). The suspended cells were homogenized with 5 strokes of a glass-teflon homogenizer and spun at 8,000g for 5 min. The membrane pellet was washed once in ice-cold phosphate-buffered saline, respun, and then suspended in either 100 μl phosphate-buffered saline containing 0.1% BSA and 100 ng/μl ^{125}I-EGF for covalent coupling or 45 μl 20 mM Hepes, pH 7.4, containing 0.1% BSA and 60 ng EGF for phosphorylation. The membranes containing ^{125}I-EGF for covalent coupling were incubated for 4 hr, after which they were washed three times with phosphate-buffered saline at 37°C and the final pellet was suspended in 0.15 ml of 20 mM Hepes containing 1 mM EDTA. Membranes to be phosphorylated were incubated at room temperature for 5 min, cooled on ice, and then incubated 10 min with 15 μl of 20 mM Hepes, pH 7.4, containing 4 μCi [γ-^{32}P]ATP, 4 mM $MnCl_2$, and 40 μM vanadate. The reaction was stopped by the addition of 0.1 ml of 0.1 M EDTA. To label EGF receptors with [2-^{3}H]mannose (**lanes E–H**) or L-[^{35}S]methionine (**lanes M–O**) the cells were incubated for 18 hr in 0.2 ml glucose-free DM containing 10% CS and 25 μCi [2-^{3}H]mannose or 0.2 ml methionine-free modified Eagle's medium containing 10% CS and 20 μCi L-[^{35}S]methionine, respectively. To label EGF receptors with $^{32}P_i$ (**lanes P–R**), the cells were incubated 4 hr in phosphate-free DM containing 0.1% BSA and 200 μCi $^{32}P_i$; during the last 15 min of incubation, 100 ng/ml EGF was added. Cellular membranes from the [2-^{3}H]mannose-, L-[^{35}S] methionine; and $^{32}P_i$—labeled cells were collected as described above. Labeled fragments of the EGF receptor (lanes B–D, F–H, J–L, N, O, Q, and R) were generated by incubating labeled membranes with 50 μg/ml trypsin-TPCK for 15 min at 25°C. The reaction was stopped and the membranes were solubilized by the addition of 1 ml RIPA containing 2 mM iodoacetate and 0.2 mg/ml phenyl methyul sulfonyl fluoride. After incubation for 20 min at 0°C, insoluble material was removed by microfuging for 5 min. To equal aliquots either trichloroacetic acid (final concentration = 10%; lanes B, F, and J), 3 μl antiserum to the EGF receptor (lanes C, G, K, N, and Q), or 3 μl normal serum (lanes D, H, L, O, and R), was added, and the mixtures were incubated 4 hr at 0°C (trichloroacetic acid) or 1 hr at 25°C (sera). The samples incubated in 10% trichloroacetic acid were microfuged 5 min; the pellet was washed twice in ice-cold acetone and suspended in 0.1 ml Laemmli buffer. To the samples incubated with serum, 30 μl 10% fixed *Staphylococcus aureus* were added, incubated 15 min, spun, washed, and solubilized in 0.1 ml Laemmli buffer. Aliquots of untrypsinized membranes (lanes A, E, and I) containing one-fourth as much portein as used for the trypsinized samples were solubilized and precipitated with 10% trichloroacetic acid as described above. The samples were electrophoresed on a 5–15% gradient gel. The gel was impregnated with fluorographic reagent and dried as described in Methods. The film was exposed for 5 days at −70°C.

moiety of the EGF/EGF receptor complex. (If the antiserum recognized EGF but not receptor fragments, it would not be expected to be able to detect biosynthetically produced receptor fragments.) Furthermore, this experiment helps delineate which portions of the EGF receptor the antiserum recognizes. Lanes C, G, K, N, and Q (Fig. 6) show immunoprecipitates of the EGF receptor which was predigested with trypsin. The ^{125}I-EGF, [^{3}H]mannose, and [^{35}S]methionine fragments had an M_r of 130k. No ^{32}P label could be observed at the corresponding location (lane J), indicating that the different isotopes labeled different segments of the EGF receptor. The ^{32}P-labeled fragments migrated at an M_r = 26k, 24k, 16k, and 13k (lane J) and most likely represent the carboxyl end of the receptor containing Tyr 1,173 [4]. These fragments were not recognized by antiserum 451 (lane K). The antiserum also did not recognize fragments of ^{32}P biosynthetically labeled receptors (lane Q) which should have also included phosphorylated serine and threonine residues [4]; thus this antiserum does not recognize the entire intracellular portion of the EGF receptor. Nevertheless, this antiserum does recognize the extracellular portion of the EGF receptor, and this is the portion which is covalently linked to ^{125}I-EGF. Since the antiserum identified biosynthetically labeled fragments produced in vitro it seems likely that it would also identify fragments produced by the cell.

DISCUSSION

Several structural details of the EGF receptor have been described. It is a 170k transmembrane protein with an extracellular domain of the receptor (which binds EGF) of approximately 104k when the contribution of the carbohydrate moieties of the molecule are included (calculated from the data in ref. 25). The intracellular 62k nonglycosylated domain of the receptor is posttranslationally modified by the addition of phosphate groups. The intracellular domain also has a kinase activity that phosphorylates proteins on tyrosine residues, including some of its own [4]. Some site on the receptor also must provide a signal for clustering [4]; however, the location of this site is unknown. Limited proteolysis can release the autophosphorylated tail of the protein (approximately 20k), the protein kinase activity (approximately 42K as well as other fragments [4]. These fragments may serve as non-membrane-bound intracellular messengers.

In this study degradation of the EGF receptor in two cell types was compared by using a variety of labeling techniques. The A-431 cell line is derived from an epidermoid carcinoma and has a very large number (approximately 10^6 per cell) of EGF receptors [26]. Human fibroblasts, on the other hand, have a more average number of EGF receptors per cell (approximately 10^5 per cell) and exhibit receptor down regulation [6]. By using highly oxidized, iodinated EGF, which covalently couples to its receptor, similar degradation products were observed in the two cell types. The A-431 fragments migrated somewhat slower in the polyacrylamide gel, which may be a consequence of differing glycosylation of the receptor in the two cell types [23].

Degradation products identified by the ^{125}I-EGF covalent coupling method were similar to those produced in vitro by using a variety of proteases [27], indicating that there are certain proteolytically susceptible loops of the receptor. The effect of in vitro proteolysis on EGF receptor activities (i.e., the EGF binding and tyrosyl kinase) has previously been examined [28–30]. The 150,000-dalton form of the receptor maintains both binding and kinase activities but has lost its highly phosphorylated segment. The 130,000 molecular weight fragment of the receptor has lost its kinase activity, which is

partially recovered in a 42,000-dalton fragment which has 28% of the activity of the undegraded EGF receptor. Either the highly phosphorylated tail or the released protein kinase fragment of the receptor are attractive candidates for second messengers of the receptor. With biosynthetically incorporated labels, stable degradation products of the receptor were not observed from either cell type. The radiolabeled precursors used in this study are distributed in the receptor molecule in distinctly different ways. Radioactive mannose becomes incorporated into the carbohydrate moieties of the receptor and thus preferentially labels the outer face of the receptor. Radioactive phosphate is transferred to several sites on the inner face of the receptor including serine, threonine, and tyrosine moieties by cytoplasmic enzymes [4] and thus preferentially labels the inner face of the receptor. Radioactive methionine is distributed throughout the entire molecule. Each of these precursors has its advantages and disadvantages. With methionine, although most degradation products would be radioactive, each fragment would have a low molar amount of radioactivity as compared to the undegraded receptor. With mannose or phosphate, not all degradation products would be labeled but those which were would be likely to have a comparatively higher amount of radioactivity per molecule. The use of these different labels should have revealed the presence of stable degradation products if they existed. Our ability to detect fragments of the EGF produced by proteases in vitro (with the exception of ^{32}P-labeled fragments) provides further evidence for this conclusion.

Since it has previously been shown that the EGF receptor is degraded in response to EGF binding, degradation products must exist. We suggest, therefore, that they must be highly unstable and therefore present in very low quantities. This would be an analogous situation to the biosynthesis of the EGF receptor where only one intermediate form was identified, using the same techniques as described in this paper [31], although many partially synthesized amino acid chains should be present, albeit at very low levels. When the cells were incubated at 20°C rather than at 37°C, a 150,000-molecular-weight fragment was observed. This fragment may conceivably be produced in skin, which is a major target tissue for EGF and tends to be cooler than the rest of the body. Several pieces of evidence indicate that the protease responsible for producing this cleavage is calpain. First, this is the major protease responsible for clearing the EGF receptor to a 150k form when cells are broken in the presence of calcium [32]. Second, EGF increases both calcium uptake and phosphatidylinositol turnover and both are substances which activate calpain [33,34].

In conclusion, covalent coupling of a hormone to its receptor is a very useful technique; however, the covalent complex may be processed by the cell in a manner different from the unmodified receptor. Thus, with the aid of biosynthetic precursors of the EGF receptor, no EGF-elicited fragments of the EGF receptor could be detected at 37°C. At 20°C, the cells could be forced into accumulating a 150k fragment. The presence of a 150k fragment indicates that calpain may play a role in the intracellular degradation of the EGF receptor.

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